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Free Radical Biology & Medicine 43 (2007) 1322–1327
www.elsevier.com/locate/freeradbiomed

Original Contribution

Interference by Mes [2-(4-morpholino)ethanesulfonic acid] and related buffers with phenolic oxidation by peroxidase

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Received 23 February 2007; revised 20 July 2007; accepted 24 July 2007
Available online 3 August 2007

Abstract

While characterizing the kinetic parameters of apoplastic phenolic oxidation by peroxidase, we found anomalies caused by the Mes [2-(4-morpholino)ethanesulfonic acid] buffer being used. In the presence of Mes, certain phenolics appeared not to be oxidized by peroxidase, yet the oxidant, H_2O_2, was utilized. This anomaly seems to be due to the recycling of the phenolic substrate. The reaction is relatively inefficient, but at buffer concentrations of 10 mM or greater the recycling effect is nearly 100% with substrate concentrations less than 100 μM. The recycling effect is dependent on substrate structure, occurring with 4′-hydroxyacetophenone but not with 3′,5′-dimethoxy-4′-hydroxyacetophenone (acetosyringone). Characterization of the reaction parameters suggests that the phenoxyl radical from the peroxidase reaction interacts with Mes, causing the reduction and regeneration of the phenol. Similar responses occurred with related buffers such as Hepes [4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid] and Pipes [piperazine-1,4-bis(2-ethanesulfonic acid)]. Results from this work and other reports in the literature indicate that great care is required in interpreting any results involving these buffers under oxidizing conditions.
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Keywords: Peroxidase; Phenol; Mes; Hepes; Pipes; Hydroxyacetophenone; Buffer anomalies; Phenolic regeneration; Free radicals

Ideally buffers maintain the pH of an environment without interfering with the chemistry or physiology of the system. Because inorganic or organic salts often interfere with biological processes under study, a series of synthetic zwitterionic buffers, such as Mes, Hepes, and Pipes, was developed for biological studies [1,2]. Although these buffers have served well for biological studies, occasionally interference is found which is not attributed to their buffering capacity. For example, Mes affects hydroponically grown cucumber, generally having a negative effect on various growth-related parameters [3]. One process that seemed to be affected was an increased oxidation of Mn^{2+} to Mn^{3+} in the nutrient solution. When used as a plant culture supplement, Mes has been reported to promote the growth of external hyphae of arbuscular mycorrhizal fungi [4] or increase the initiation of embryogenic tissue in loblolly pine [5]. High concentrations, >0.4 M, of Mes and Pipes buffers strongly promote polymerization of purified tubulin [6,7]. In a H_2O_2-dependent Ames test for mutagenicity, Mes buffer was able to protect an Ames tester *Salmonella* strain from mutagenesis by benzidine derivatives [8]. A detailed mechanistic study demonstrated that Hepes buffer interferes with studies of peroxynitrite by reacting with it to form hydrogen peroxide [9,10]. Often these observations go unpublished [4] unless they help understand the system being studied or they interfere with universal assays, such as the protein determination [11,12].

During investigations of plant phenolics as substrates for peroxidase we observed that the Mes buffer was interfering with the reaction. Specifically, hydroxyacetophenone, a phenolic

Abbreviations: Hepes, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; Mes, 2-(4-morpholino)ethanesulfonic acid; Pipes, piperazine-1,4-bis(2-ethanesulfonic acid).

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0891-5849/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.freeradbiomed.2007.07.020

found in the apoplast of tobacco [13], was not consumed during peroxidase reactions when Mes was used as a pH buffer. Here we report and investigate the mechanism of this interference by Mes. After observing the effects of various parameters on this interference, we suggest that Mes, as well as other morpholine/piperazine buffers, may be causing the redox recycling of certain phenolic substrates of peroxidase.

Materials and methods

Chemicals

Horseradish peroxidase (P-8250), 4-(2-aminoethyl)morpholine (A-55004), acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone, D-134406), 4-(2-hydroxyethyl)morpholine (H-28203), 4′-hydroxyacetophenone (278564), Mes (M-3058), Hepes (H-3784), and Pipes (P-2949) were purchased from Sigma–Aldrich Chemicals, Inc. (St. Louis, MO, USA).

Peroxidase reactions

Enzyme reactions, 10 ml, were carried out in a shaking water bath, at 27 °C. The typical composition was 0.72 U/ml peroxidase, 100 μM H_2O_2, 50 μM hydroxyacetophenone, and 25 mM Mes, pH 6.0. Aliquots were periodically removed for analysis. Peroxidase activity was measured in units as described by Sigma; one unit (U) will oxidize 1 μmol of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)/min.

HPLC-UV quantification of phenolics

Acetosyringone or hydroxyacetophenone was separated and quantified using an Onyx monolith C18 analytical column, 100×4.6 mm i.d. (Phenomenex, Torrance, CA, USA), with a Waters (Milford, MA, USA) quaternary pump, autosampler, photodiode array detector, and Empower data acquisition on a Dell Pentium 4 computer. Aliquots, 150 μl, of samples were acidified with phosphoric acid (0.1%) and placed in the autosampler using a 10-μl injection volume. An isocratic mobile phase of 30% methanol in 0.01% aqueous phosphoric acid, 2 ml/min, separated the phenolics within 4 min. Quantification using peak height was performed using the UV_{max} wavelength for each peak, acetosyringone, 300 nm, hydroxyacetophenone, 276 nm, and calibration with standards.

FOX2 (ferrous oxidation in xylenol orange) assay for hydrogen peroxide

In this spectrophotometric method, ferrous ions are oxidized by hydrogen peroxide to ferric ions, which bind with xylenol orange to give a colored complex with increased absorbance at 560 nm [14]. The advantage of this technique is that it does not rely on peroxidase, which is affected by the exogenous phenolics added during this study. The FOX2 reagent contains 125 μM xylenol orange, 250 μM ammonium ferrous sulfate, and 4.4 mM butylated hydroxytoluene in 90% methanol containing 25 mM sulfuric acid. Using a 96-well plate, 270 μl of FOX2 reagent was added to 30-μl samples. After 30 min incubation, the plates were read at 560 nm using a Molecular Devices Versamax microplate reader (Sunnyvale, CA, USA). The absorbance change was compared to hydrogen peroxide standards.

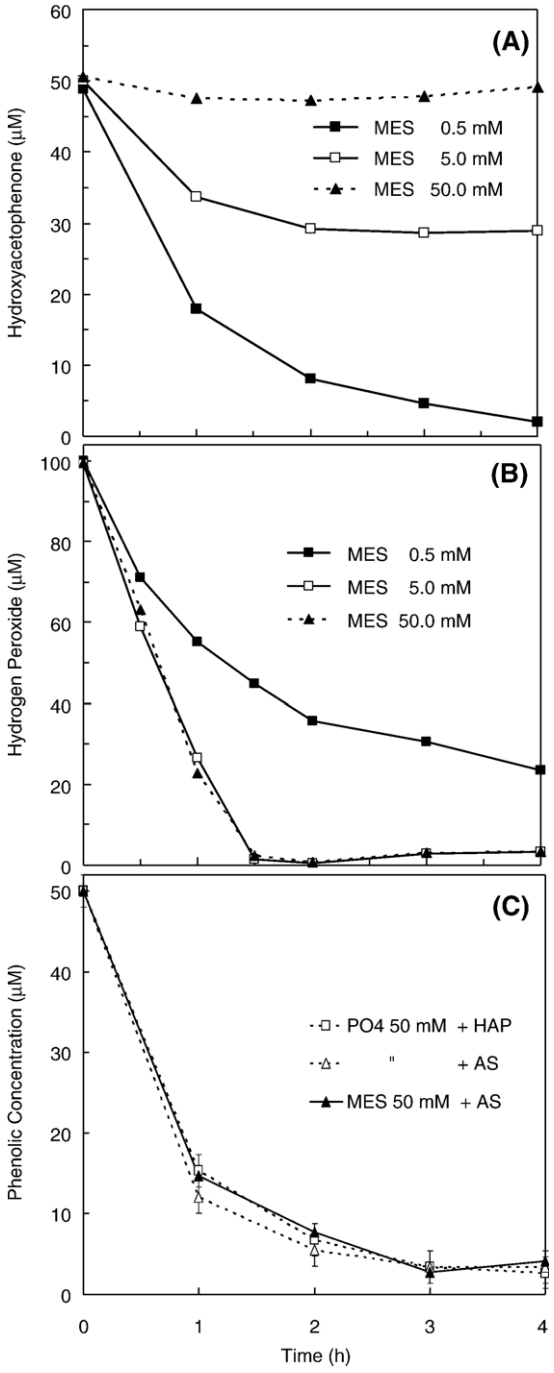

Fig. 1. The effect of Mes on the utilization of hydroxyacetophenone and H_2O_2 by peroxidase. Hydroxyacetophenone (HAP), 50 μM, was incubated with H_2O_2, 100 μM, and horseradish peroxidase, 0.72 U/ml, in different concentrations of Mes buffer, pH 6.0. (A) The concentration of HAP was determined by HPLC-UV and (B) the concentration of H_2O_2 was determined spectrophotometrically using the FOX2 assay. (C) Similar reaction parameters were used to compare the effect of sodium phosphate buffer, pH 6.0, with HAP. Also the effect of high concentrations of these buffers with acetosyringone (AS) was compared. See Materials and methods for further details.

Results

Effect of increased Mes buffer concentration on phenolic oxidation by peroxidase

Studies of apoplastic phenolics as substrates for peroxidase revealed that the Mes buffer being used interfered with the reaction of some phenolics. The oxidation of hydroxyacetophenone by peroxidase and H_2O_2 was carried out with different concentrations of Mes buffer, pH 6 (Fig. 1). The final amount of hydroxyacetophenone oxidized in reactions decreased as the Mes concentration increased from 0.5 to 50 mM and in the latter case was negligible (Fig. 1A). Conversely, the amount of H_2O_2 utilized in reactions containing higher Mes concentrations, 5 or 50 mM, was greater than in reactions using only 0.5 mM Mes (Fig. 1B). This suggested that the increased Mes concentration was not inhibiting the reaction but was interfering in some other way.

The negligible oxidation of hydroxyacetophenone with high buffer concentration did not occur when 50 mM phosphate buffer, pH 6.0, was used (Fig. 1C), suggesting that Mes itself was interfering. Also this interference by Mes was substrate specific because it did not occur when another apoplastic phenolic, acetosyringone, was used under similar conditions. The oxidation of acetosyringone in either 50 mM Mes or 50 mM phosphate buffer, pH 6.0, was similar (Fig. 1C).

Effect of varying reaction parameters on the reaction kinetics

To better understand what was occurring with Mes in peroxidase reactions with hydroxyacetophenone, other reaction parameters were investigated. The typical reaction mixture included 0.72 U/ml peroxidase, 100 μM H_2O_2, 50 μM hydroxyacetophenone, and 25 mM Mes, pH 6.0, unless otherwise indicated. As indicated in Fig. 1, increasing the Mes concentration in the reaction mixtures decreased the apparent rate of hydroxyacetophenone oxidation, which under these conditions was negligible at Mes concentrations of 25 mM or greater (Fig. 2A). Conversely, in the same reaction mixtures, the increase in Mes concentration to 5 mM and greater increased the rate of H_2O_2 utilization to a maximum level.

When the hydroxyacetophenone concentration was varied in these reaction mixtures from 50 to 200 μM, the rate of H_2O_2 utilization increased (Fig. 2B). The increase in the rate of H_2O_2 utilization was almost linear to the increase in

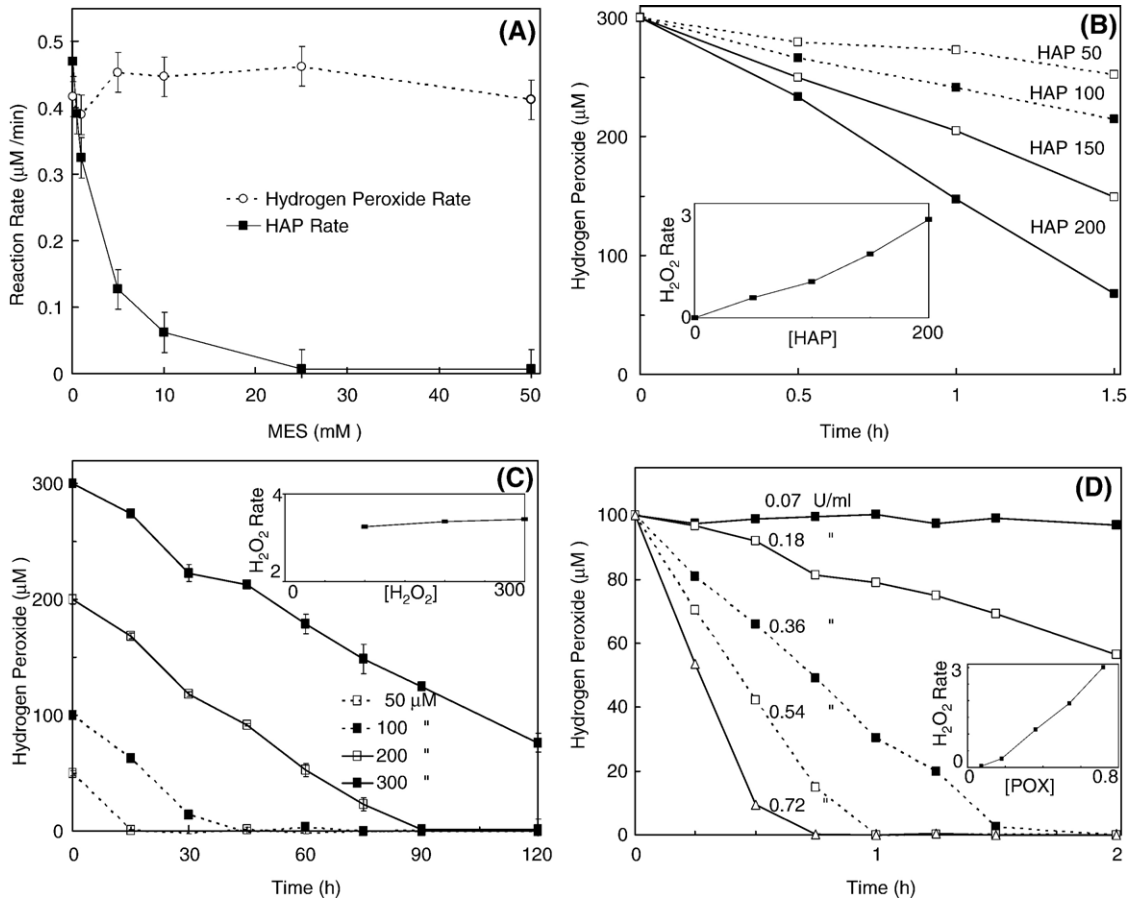

Fig. 2. Effect of modifying various reaction parameters on the reaction rate of oxidation of HAP by peroxidase. Unless otherwise indicated, the general enzyme reaction included H_2O_2, 100 μM; HAP, 50 μM; peroxidase, 0.72 U/ml; in 25 mM Mes, pH 6.0. (A) Effect of Mes concentration on the reaction rate of HAP and H_2O_2 utilization; peroxidase concentration, 0.36 U/ml. (B) Effect of HAP concentration on the utilization and reaction rate of H_2O_2, 300 μM. (C) Effect of H_2O_2 concentration on the utilization and reaction rate of H_2O_2. (D) Effect of peroxidase on the utilization and reaction rate of H_2O_2. See Materials and methods for further details.

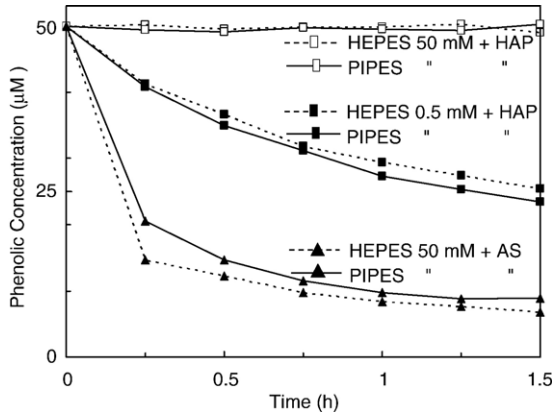

Fig. 3. Effect of structurally related buffers, Hepes and Pipes, on phenolic oxidation by peroxidase. Hydroxyacetophenone or acetosyringone, 50 μM, was incubated with H_2O_2, 100 μM, and horseradish peroxidase, 0.72 U/ml, in either Hepes or Pipes buffer, pH 6.0. The concentration of the phenolics was monitored by HPLC-UV. See Materials and methods for other details.

hydroxyacetophenone concentration (Fig. 2B, inset). Because these reactions were carried out in 25 mM Mes, as mentioned above, the different initial hydroxyacetophenone concentrations remained constant throughout the reaction (data not shown).

Increasing the initial H_2O_2 concentration in the typical reaction mixture from 50 to 300 μM had little effect on the rate of H_2O_2 utilization (Fig. 2C, inset). Consistent with this, within individual reactions as the hydrogen peroxide was utilized and its concentration decreased, the rate of H_2O_2 utilization remained nearly constant until it was depleted (Fig. 2C).

Increasing the peroxidase concentration from 0.18 to 0.72 U/ml increased the rate of H_2O_2 utilization almost linearly; reaction rates with lower enzyme concentrations were not easily detected under these conditions (Fig. 2D, inset).

Results with structurally related buffers and compounds

We found similar results when we used Hepes and Pipes buffers, which are structurally related to Mes. At low buffer concentrations of 0.5 mM Hepes or Pipes, pH 6.0, hydroxyacetophenone was oxidized in the presence of H_2O_2 and peroxidase (Fig. 3). However, at higher buffer concentrations of 50 mM Hepes or Pipes, pH 6.0, hydroxyacetophenone appeared to be recycled and did not decrease in concentration (Fig. 3). As with Mes, high concentrations of these buffers did not interfere with oxidization of acetosyringone under these conditions.

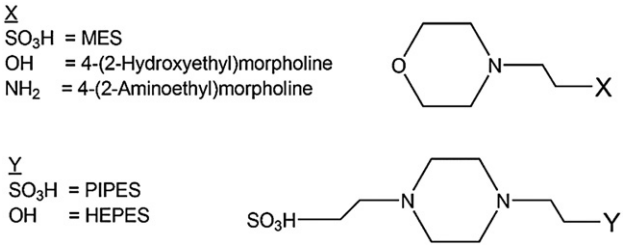

Fig. 4. Molecular structures of Mes and related compounds that interfere with hydroxyacetophenone oxidation.

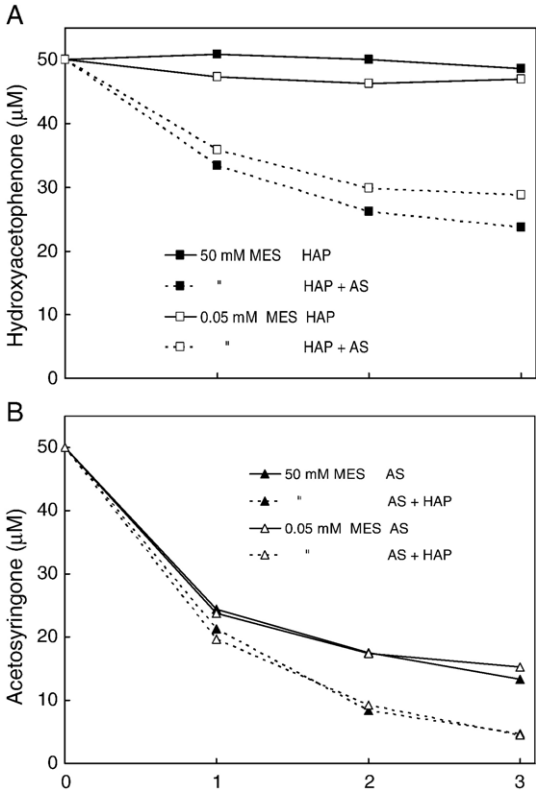

Fig. 5. The effect of Mes on co-oxidation of hydroxyacetophenone and acetosyringone. Hydroxyacetophenone (HAP) and/or acetosyringone (AS), 50 μM, was incubated with H_2O_2, 100 μM, and horseradish peroxidase, 0.072 U/ml, in different concentrations of Mes buffer, pH 6.0. The concentration of (A) hydroxyacetophenone or (B) acetosyringone was determined by HPLC-UV. See Materials and methods for further details.

Using other structurally related compounds (Fig. 4), aminoethyl morpholine and hydroxyethyl morpholine, we also found similar interference with the hydroxyacetophenone oxidation by peroxidase (data not shown). One common structural feature of these compounds is the trisubstituted N (Fig. 4), which could

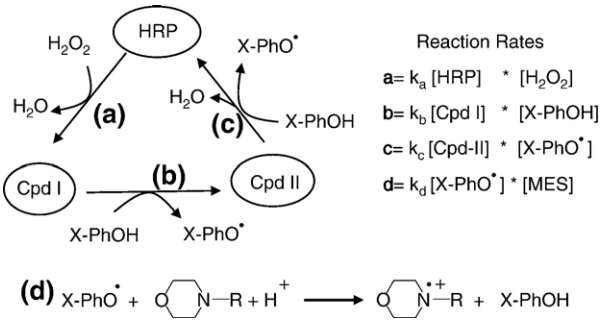

Fig. 6. Proposed mechanism of Mes interference with peroxidase oxidation of hydroxyacetophenone. (a) The native peroxidase enzyme (HRP) loses 2 electrons to H_2O_2, producing water, and incorporates the remaining oxygen atom to form compound I (Cpd I). (b) Cpd I can gain an electron from hydroxyacetophenone (X-PhOH) producing compound II (Cpd II) and phenoxyl radical (X-PhO•). (c) Cpd II will repeat the latter process reverting back to its native state (HRP). (d) Mes seems to be able to recycle (inefficiently) the phenoxyl radical back to hydroxyacetophenone, which would likely oxidize Mes with the loss of an electron.

support the loss of an electron to recycle the phenoxyl radical back to hydroxyacetophenone.

Effects of Mes on co-oxidation of phenolics

Co-oxidation can be an important process in the plant apoplast, where multiple bioactive phenolics are present during periods of oxidative stress when hydrogen peroxide and peroxidase are present [15,16]. In a previous study we demonstrated that co-oxidation of hydroxyacetophenone and acetosyringone occurred in the presence of stressed soybean suspension cells. To determine if high concentrations of Mes would affect co-oxidation of these phenolics we examined the net loss of each using 0.5 mM and 50 mM Mes (Fig. 5). Due to the increased reaction rate of co-oxidation a lower amount of peroxidase, 0.1 U/ml, was used. The increased rate of oxidation when both hydroxyacetophenone and acetosyringone were present was not affected by the Mes concentration.

Discussion

This study shows that Mes at concentrations of 5 mM and greater can interfere with the oxidation of certain phenolics. Hydroxyacetophenone was oxidized by peroxidase and hydrogen peroxide yielding a phenoxyl radical, which presumably reacted with Mes and was recycled back to hydroxyacetophenone. Structurally related compounds such as Pipes and Hepes were found to have a similar recycling effect on hydroxyacetophenone oxidation. This recycling effect by Mes was substrate dependent and did not occur with acetosyringone. Mes did not affect phenolic co-oxidation, which is more prevalent in biological situations, such as the plant apoplast, where multiple phenolics are present under oxidative conditions.

The model proposed in Fig. 6 explains the effects of varying reactant concentrations on the utilization of hydrogen peroxide and hydroxyacetophenone. The first three reactions describe standard peroxidase chemistry in which peroxidase catalyzes the single-electron oxidation of phenolic substrates. Varying the concentration of H_2O_2 (Fig. 2C) had little effect on the reaction rate as indicated both by increasing the initial H_2O_2 concentration of the reactions and by the relatively constant rate of H_2O_2 utilization even as the H_2O_2 concentration was nearly consumed. Reaction (a) is known to be much faster than either reaction (b) or reaction (c), which are the rate-limiting reactions. Because the concentrations of the reactants in reactions (b) and (c) are relatively constant due to the recycling of hydroxyacetophenone, the overall reaction velocity is relatively constant until the H_2O_2 concentration is depleted. Increasing the concentration of either hydroxyacetophenone (Fig. 2B) or peroxidase (Fig. 2D) would increase the rate-limiting reactions (b) and (c) and therefore increase H_2O_2 utilization.

The phenoxyl radical derived from 4-hydroxyacetophenone in reactions (b) and (c) can abstract a hydrogen atom from Mes, when the latter is present in high concentrations, to regenerate hydroxyacetophenone [reaction (d)]. The reaction of Mes and other amine-based buffers with hydroxyacetophenone but not with acetosyringone can be explained by the reduction potentials of the corresponding phenoxyl radicals. The phenoxyl radical from 4-hydroxyacetophenone has a potential ($E°$) about 1 V vs a normal hydrogen electrode [17], which is apparently high enough to enable the hydrogen abstraction from Mes. The two electron-donating methoxy substituents on acetosyringone lower the reduction potential of the phenoxyl radical as well as increasing the steric hindrance around the phenoxyl radical center, preventing significant Mes oxidation by this radical.

Reaction (d) seems to be inefficient, requiring the Mes concentration to be 5 to 25 mM (Fig. 2A) for noticeable recycling of the phenoxyl radical. The radical derived from Mes can form other stable oxidized products analogous to the examples described for Pipes and Hepes [18].

Hepes and Pipes have been reported to have important effects in reactions involving other radicals, such as peroxynitrite, which generates $NO^{\bullet}$ and the superoxide anion radical $O_2^{\bullet -}$ [9,10,18]. Kirish et al. carried out a detailed study proposing that Hepes and related tertiary amines could be oxidized by strong oxidants yielding radical cations. They demonstrated that the radical cations resulting from Hepes oxidation reacted with molecular oxygen, which accounted for the high level of H_2O_2 found in their system when peroxynitrite was present. It is likely that a similar situation in which hydrogen peroxide is produced from the Mes radical cations occurs in our system.

When used in relatively high concentrations as buffers in biological systems, compounds such as Mes and Hepes can function as radical scavengers of certain phenolic radicals. It is important to be aware that they may cause subtle changes in the profile of redox-sensitive secondary metabolites. Because these secondary metabolites can have important bioactive functions, this could lead to changes in the biological behavior of the system under study as noted earlier [3,4,6,8].

Acknowledgments

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